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Nasopharyngeal Microbiota Profiles in Rural Venezuelan Children Are Associated With Respiratory and Gastrointestinal Infections

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Background. Recent research suggests that the microbiota affects susceptibility to both respiratory tract infections (RTIs) and gastrointestinal infections (GIIs). In order to optimize global treatment options, it is important to characterize microbiota profiles across different niches and geographic/socioeconomic areas where RTI and GII prevalences are high.

Methods. We performed 16S sequencing of nasopharyngeal swabs from 209 Venezuelan Amerindian children aged 6 weeks–59 months who were participating in a 13-valent pneumococcal conjugate vaccine (PCV13) study. Using random forest models, differential abundance testing, and regression analysis, we determined whether specific bacteria were associated with RTIs or GIIs and variation in PCV13 response.

Results. Microbiota compositions differed between children with or without RTIs ($P = .018$) or GIIs ($P = .001$). Several species were associated with the absence of infections. Some of these health-associated bacteria are also observed in developed regions, such as *Corynebacterium* ($\log_2(\text{fold change [FC]}) = 3.30$ for RTIs and $\log_2(\text{FC}) = 1.71$ for GIIs), while others are not commonly observed in developed regions, such as *Acinetobacter* ($\log_2(\text{FC}) = 2.82$ and $\log_2(\text{FC}) = 5.06$, respectively). *Klebsiella* spp. presence was associated with both RTIs ($\log_2(\text{FC}) = 5.48$) and GIIs ($\log_2(\text{FC}) = 7.20$).

Conclusions. The nasopharyngeal microbiota of rural Venezuelan children included several bacteria that thrive in tropical humid climates. Interestingly, nasopharyngeal microbiota composition not only differed in children with an RTI but also in those with a GII, which suggests a reciprocal interplay between the 2 environments. Knowledge of region-specific microbiota patterns enables tailoring of preventive and therapeutic approaches.

Keywords. respiratory microbiota; children; rural; infections.

Despite progress in the reduction of global childhood mortality over the past 2 decades, respiratory tract infections (RTIs) and gastrointestinal infections (GIIs) remain the leading causes of death in children aged <5 years worldwide, particularly in regions marked by socioeconomic inequality, poor sanitation, inadequate nutrition, and low public health services coverage [1]. When it comes to treatment, medical practitioners apply a “one microbe one disease” model. However, recent studies

suggest that the microbiota (the collective abundance of bacterial species that inhabit the body) influences susceptibility to and outcome of both GIIs and RTIs [2–4]. In this context, the composition of the gut microbiota has received considerable attention due to its significant role in mucosal barrier function and healthy immune maturation in childhood [4]. Likewise, however, recent studies have revealed the important role of the respiratory microbiota in maintaining respiratory health [5]. While many observational studies and trials have assessed the influence of the intestinal microbiota on respiratory conditions [6–8], the reverse is much less studied.

Venezuela is a South American country with a rich cultural display and a large variation in the level of urbanization between regions. Warao Amerindians inhabit the Orinoco Delta and are the second largest indigenous group in Venezuela. Around one-third of Warao children die before the age of 12 years; the majority of deaths (97%) occur before age 5 years. RTIs and GIIs are the 2 most common causes of mortality in these children (18% and 63%, respectively), as previously described

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[9–11]. Although the respiratory microbiota has been increasingly recognized as a mediator of disease susceptibility over the past decade, most microbiota studies have been carried out in Westernized populations. A comprehensive understanding of the link between RTIs and GIIs and the composition of the microbiota in vulnerable rural populations is necessary to develop region-specific preventive and therapeutic strategies.

Further, an association between the gut microbiota and a range of vaccine responses has been described [12]. Immunization currently prevents between 2 million and 3 million deaths due to infectious diseases every year [13]. However, most vaccines do not convey 100% protection, and several factors can influence the strength of the immune responses mounted upon vaccination in children, such as age, season, and nutritional status [14]. The 13-valent pneumococcal conjugate vaccine (PCV13) was integrated in the Venezuelan national immunization program in 2014 due to high carriage rates of *Streptococcus pneumoniae* [10]. However, studies showed that protective antibody levels after PCV13 introduction in vulnerable indigenous children differed from those observed in studies in healthy non-native children [15, 16]. Understanding the potential links between the respiratory microbiota and PCV13 response will help to address this discrepancy.

In this study, we used 16S sequencing of nasopharyngeal samples of indigenous Venezuelan children in order to characterize the nasopharyngeal microbiota of children living in rural conditions, assess the association between the nasopharyngeal microbiota and the presence of RTIs and GIIs, and study the relationship between the prevaccination nasopharyngeal microbiota and postvaccination PCV13 antibody titers. Because early-life microbial dysbiosis has been linked to subsequent susceptibility to infectious diseases [17], we studied only children aged <5 years.

METHODS

Study Population

We conducted a cross-sectional study to examine microbiota community profiles and the association between bacterial taxa and RTIs/GIIs combined with a prospective cohort study to examine the relationship between nasopharyngeal microbiota profiles at inclusion and antibody levels in serum after completion of a primary PCV13 vaccination series. Microbiota samples were collected between 23 May 2012 and 7 July 2012 from children aged <5 years who participated in a PCV13 study [16] in the Venezuelan Orinoco River Delta. Further details about the study population can be found in the [Appendix](#). The Instituto de Biomedicina Ethical Committee (Caracas, Venezuela), the Delta Amacuro Indigenous Health Office, and community leaders approved the study.

Data Collection and Sampling

A team of physicians and medical students approached children from 9 Warao communities. The presence of an RTI at

the time of sampling was defined as symptoms and signs of an upper (rhinorrhea, pharyngitis, sinusitis) and/or lower (bronchitis, bronchiolitis, pneumonia) respiratory tract infection according to previously described methods [9]. The presence of a GII at the time of sampling was defined as current diarrhea with or without other symptoms involving the gastrointestinal tract (such as vomiting or abdominal distension). Nasopharyngeal samples were obtained before administration of the first dose of the PCV13 vaccine using a flexible swab (Copan, Italia) in skim milk tryptone glucose glycerol medium [18]. Swabs were subjected to 1 freeze–thaw cycle to allow 300 µL to be sent to our laboratories for further processing [19].

Both swabs and serum samples were refrigerated at 4°C for ≤3 days before being transported to a –20°C freezer and, within 4 weeks, to a –70°C freezer. Pneumococcal serotype-specific serum antibody concentrations were determined using a fluorescent bead-based multiplex immunoassay as described previously [20].

High-throughput Sequencing of Nasopharyngeal Samples

Bacterial DNA was isolated and polymerase chain reaction amplicon libraries of the 16S ribosomal RNA gene were generated by amplifying the V4 hypervariable region as previously described [3]. The pooled libraries were sequenced on the Illumina MiSeq platform (Illumina Inc, San Diego, CA). See the [Appendix](#) for a detailed description of laboratory procedures and bioinformatic processing, including validated methods to identify and remove possible contaminants (Supplementary [Tables 1](#) and [2](#)) [21]. Sequence data and R scripts are available from the Dryad data repository (doi: [10.5061/dryad.h44j0zpfq](https://doi.org/10.5061/dryad.h44j0zpfq)).

Statistical Analyses

All analyses were performed in R version 3.5.0. To identify bacterial community clusters, we used hierarchical clustering based on Bray-Curtis dissimilarity. Clusters were named after the most abundant taxa within a cluster. We determined whether microbiota compositions differed between children with or without RTIs or GIIs by calculating differences in alpha diversity between groups and by permutational multivariate analysis of variance (PERMANOVA). We tested whether certain bacterial taxa were associated with an RTI or GII using random forest and differential abundance testing using metagenomeSeq [22]. We used the mean of serotype-specific log-transformed pneumococcal antibody levels as a readout for pneumococcal vaccine response. To study the relationship between prevaccination microbiota profiles and postvaccination antibody levels, we performed a combination of random forest and linear regression analyses. *P* values were corrected using the Benjamini-Hochberg method to account for multiple testing. A detailed description of statistical analyses can be found in the [Appendix](#).

Table 1. Characteristics of the Study Population

Characteristics	Patients
Sex, n (%)	
Male	87 (46)
Female	104 (54)
Age, median (interquartile range), months	15 (7–21)
Nutritional status categories, n (%) ^a	
Well-nourished (HAZ ≥ –2 SD)	118 (62)
Stunted (HAZ < –2 SD)	69 (36)
Feeding, n (%)	
Breastfed at the moment of sampling	150 (79)
Not breastfed at the moment of sampling	41 (21)
Presence of infectious illnesses, n (%)	
Respiratory infection	103 (54)
Gastrointestinal infection	28 (15)
Antibiotic use at or 1 week prior to sampling, n (%)	15 (8)
Community, n (%)	
Araguabisi	14 (7)
Araguaimujo	33 (17)
Arature	21 (11)
Bonoina	20 (10)
Guayaboroina	8 (4)
Ibaruma	30 (16)
Jobure de Curiapo	33 (17)
Merejina	9 (5)
Winikina	23 (12)

Abbreviations: HAZ, height-for-age; SD, standard deviation.

^aFor the calculation of nutritional status, 4 children were excluded because of probable measurement errors.

RESULTS

A total of 191 children were included in the analyses. Characteristics of the study population are displayed in Table 1. A total of 36% of children showed signs of chronic malnourishment. An RTI (with upper and/or lower respiratory tract infection symptoms) was present in 54% of children, while 15% showed symptoms of a GII, with 15 children (8%) suffering from both an RTI and a GII. For microbiota analysis, we generated 2 953 950 reads (median, 10 375 reads per sample; range, 107–74 630), which were grouped into 121 operational taxonomic units (OTUs).

Microbial Community Composition of Rural Venezuelan Amerindian Children

The most abundant bacteria in our study population were *Moraxella* (1) with a mean relative abundance of 22.5%, followed by *Corynebacterium propinquum* (2) (12.8%), *Dolosigranulum* (3) (11.3%), *Haemophilus* (4) (8.1%), and *Streptococcus* (5) (4.6%). We observed 15 clusters that represented different community profiles, with 9 clusters containing 5 children or more (Figure 1). The largest cluster (n = 80 children) was dominated by *Moraxella* (1) with a within-cluster mean relative abundance of 41.2%, followed by the second largest cluster (n = 33 children), which was dominated by a combination of *C. propinquum* (2) (39.4%) and *Dolosigranulum* (3)

(29.1%). The remaining smaller clusters were dominated by *Ornithobacterium* (6) (n = 16 children, 26.6%), *Haemophilus* (4) (n = 13 children, 40.5%), *Acinetobacter* (10) (n = 9 children, 44.7%), *Klebsiella* (7) (n = 7 children, 91.4%), *Flavobacterium* (14) (n = 6 children, 22.3%), *Leuconostoc* (9) (n = 6 children, 78.0%), and *Arthrobacter* (12) (n = 5 children, 60.0%). Children in the *Klebsiella*- and *Leuconostoc*-dominated clusters were significantly more likely to have a GII than children in any other cluster (Fisher exact test: *Klebsiella* (7), *P* = .01, odds ratio [OR] = 8.7, 95% confidence interval [CI] = 1.4 to 63.3 and *Leuconostoc* (9), *P* = .042, OR = 6.3, 95% CI = .8 to 50.0), and there was a trend toward children in the *Acinetobacter*-dominated (10) cluster to be less likely to have an RTI (*P* = .083, OR = 0.2, 95% CI = .03 to 1.25).

Association of Nasopharyngeal Microbiota Profiles With the Presence of Infections

We found that microbiota profiles differed between children both with and without RTI (PERMANOVA: $R^2 = 1.2\%$, *P* = .018; Figure 2A) and between children with and without GII ($R^2 = 2.0\%$, *P* = .001; Figure 2B). Microbiota composition was further affected by age ($R^2 = 1.2$, *P* = .018; Figure 2C, Supplementary Table 3). We also saw a difference in microbial compositions between children when we divided children into 4 categories based on their combined status for both RTIs and GIIs (Supplementary Figure 4).

We measured alpha diversity to study the microbial evenness and richness within samples. Mean alpha diversity as measured using the Shannon diversity index (richness and evenness) and the Chao1 estimate of richness were 1.43 ± 0.48 standard deviation (SD) and 21.38 ± 10.00 SD, respectively. Children with a GII showed a lower Shannon index than children without a GII (linear model: $F_{1,182} = 8.5$, *P* = .004; Figure 3A, 3B), that is, their microbial profiles were less evenly distributed. Children with an RTI also showed a trend toward a lower Shannon index compared with children without an RTI (linear model: $F_{1,182} = 3.40$, *P* = .067; Figure 3C, 3D). In addition, age affected richness, with the Chao estimate (linear model: $F_{1,182} = 3.59$, *P* = .060) being lower in younger children compared with older children (Figure 3E, 3F). There were no differences in both of the measures of alpha diversity between stunted and well-nourished children (Figure 3G, 3H).

Associations of Bacterial Taxa With the Presence of Infections

We found that 7 OTUs were more abundant in children with RTIs (Table 2, Figure 4A), with *Klebsiella* (7) (log₂(fold change [FC]) = 5.48) and *Anoxybacillus* (33) (log₂(FC) = 2.25) showing the highest fold changes. On the other hand, 13 OTUs were more abundant in children without RTIs, of which *Caulobacteraceae* (18) (log₂(FC) = 4.58), *Schlegelella* (40) (log₂(FC) = 3.99), *Corynebacterium* (91) (log₂(FC) = 3.30), and *Acinetobacter* (10) (log₂(FC) = 2.82) showed the highest fold changes (Table 2, Figure 4A). Random forest analysis identified an additional

Table 2. Bacteria Discriminative for Respiratory Tract Infection Groups as Identified by metagenomeSeq and Random Forest

Operational Taxonomic Unit	Log Fold Change	P Value	Adjusted P Value	Method ^a
Associated with disease (RTI)				
<i>Bacillus</i> (25)	-1.13	.0001	.0006	Both
<i>Klebsiella</i> (7)	-5.48	<.0001	<.0001	MGS
<i>Anoxybacillus</i> (33)	-2.25	<.0001	<.0001	MGS
<i>Thermus thermophilus</i> (26)	-1.43	.0003	.0017	MGS
<i>Arthrobacter</i> (12)	-1.41	.0104	.0359	MGS
<i>Xanthobacteraceae</i> (97)	-1.40	.0000	<.0001	MGS
<i>Actinomyces</i> (57)	-1.18	.0001	.0007	MGS
Associated with health (absence of RTI)				
<i>Caulobacteraceae</i> (18)	4.58	<.0001	<.0001	Both
<i>Schlegelella</i> (40)	3.99	<.0001	<.0001	Both
<i>Pseudomonas putida</i> (49)	1.10	<.0001	.0001	Both
<i>Corynebacterium</i> (91)	3.30	<.0001	<.0001	MGS
<i>Acinetobacter</i> (10)	2.82	<.0001	.0001	MGS
<i>Acinetobacter</i> (47)	2.38	<.0001	<.0001	MGS
<i>Acinetobacter soli</i> (41)	1.78	.0001	.0007	MGS
<i>Stenotrophomonas maltophilia</i> (58)	1.40	<.0001	<.0001	MGS
<i>Pseudomonas</i> (23)	1.25	.0007	.0034	MGS
<i>Acinetobacter</i> (11)	1.15	<.0001	<.0001	MGS
<i>Wautersiella</i> (61)	1.09	.0001	.0005	MGS
<i>Corynebacterium propinquum</i> (2)	0.50	.2937	.4581	RF ^b
<i>Pseudomonas syringae</i> (27)	0.12	.5946	.7027	RF

P values have been adjusted using Benjamini-Hochberg correction for multiple testing.

Abbreviation: MGS, metagenomeSeq; RF, random forest; RTI, respiratory tract infection; VSURF, variable selection using random forests.

^aBoth methods adjusted for age.

^bVariable selection by VSURF.

2 OTUs that were associated with health (absence of RTIs), *Corynebacterium propinquum* (2) and *Pseudomonas syringae* (27) (Table 2). We identified 12 OTUs that were more abundant in children with a GII (Table 3, Figure 4B), with *Klebsiella* (7) again showing the highest fold change ($\log_2(\text{FC}) = 7.20$).

Further, *Klebsiella* (7) was also the only OTU identified as discriminative between GII groups by random forest. Of the 23 OTUs that were more abundant in children without a GII, 3 *Acinetobacter* species were among those OTUs that showed a fold change of 2 or higher (*Acinetobacter* (10): $\log_2(\text{FC}) = 5.06$,

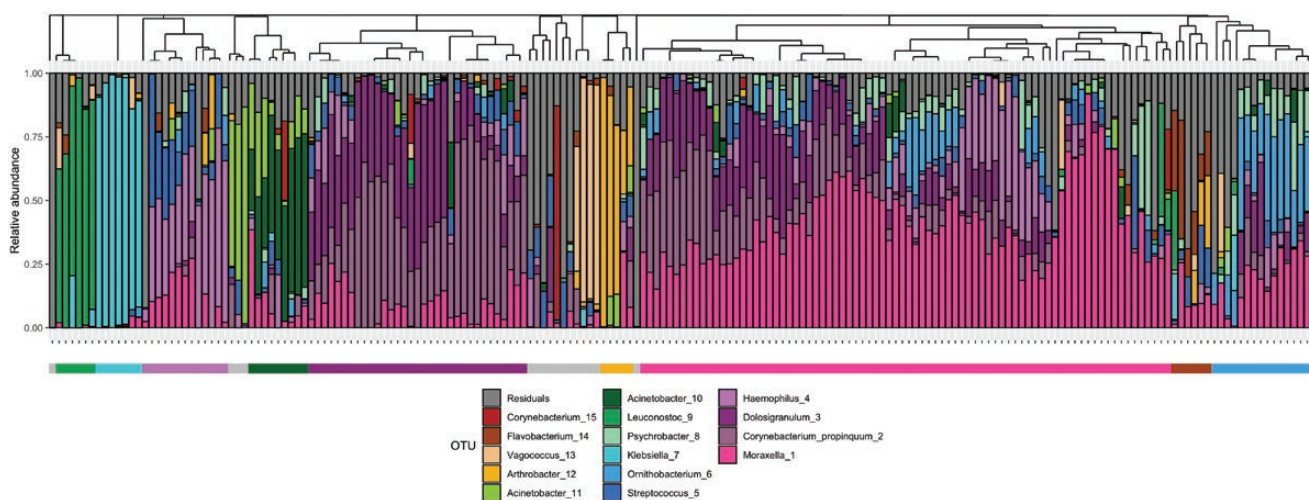


Figure 1. Cumulative relative abundance of the 15 most abundant OTUs in the samples. Each bar represents an individual sample. The dendrogram represents similarity between samples based on Bray-Curtis dissimilarity. The bar below the plot represents sample clusters after hierarchical clustering. Abbreviation: OTU, operational taxonomic unit.

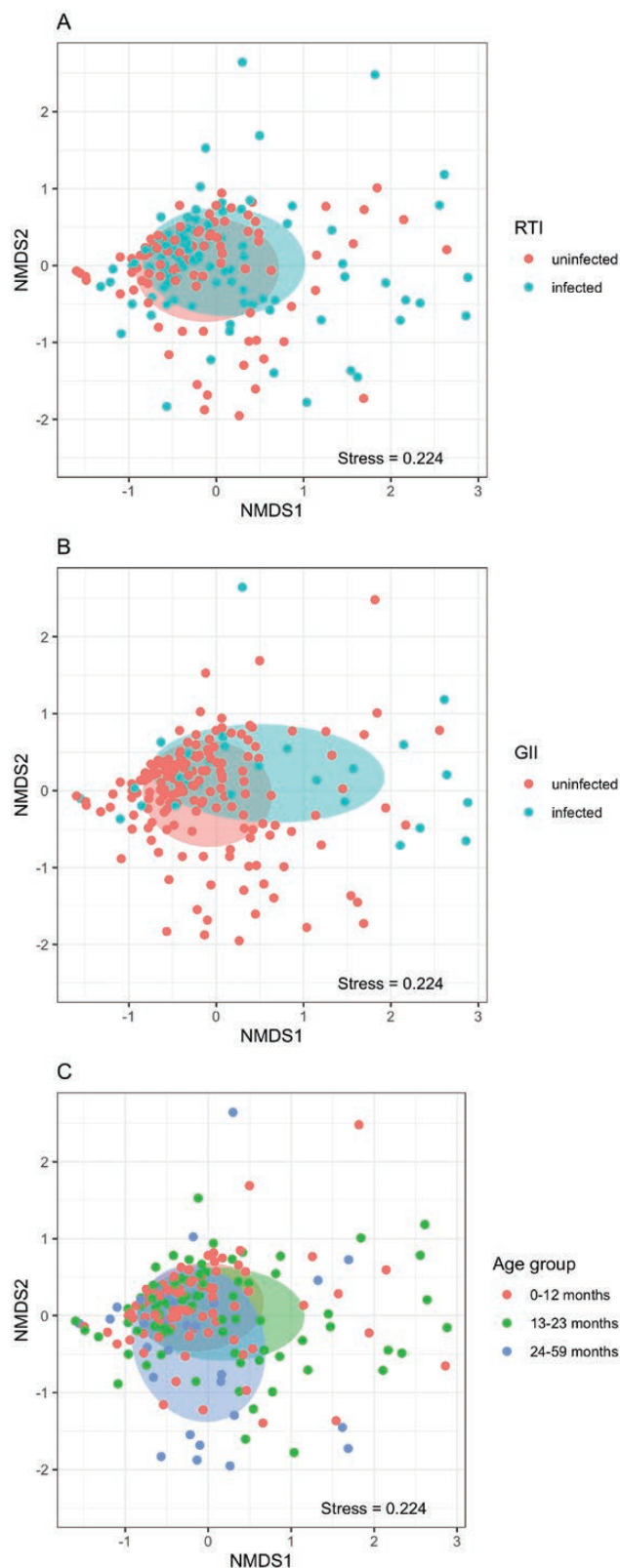


Figure 2. NMDS plots showing the difference in microbial community composition between (A) RTI groups, (B) GII groups, and (C) age groups. Abbreviations: GII, gastrointestinal infection; NMDS, non-metric dimensional scaling; RTI, respiratory tract infection.

Acinetobacter (47): $\log_2(\text{FC}) = 3.14$, *Acinetobacter soli* (41): $\log_2(\text{FC}) = 2.34$).

Association of Pre-PCV Microbiota Profiles With Post-PCV Antibody Levels

Postvaccination serum samples were collected at a median of 6.7 weeks (interquartile range, 6.3–6.9 weeks) and were available for 138/191 children. The remainder of the children were lost to follow-up, which is common in these nomadic communities. The random forest model that included OTUs from pre-PCV13 microbiota samples plus host characteristics explained 31.67% of the variance in PCV13 postvaccination response (Supplementary Figure 5). The top 15 covariates that were associated with post-PCV13 vaccine response in this model were used in the final mixed-effect model. Days to follow-up, increasing age, *Arthrobacter* (12), and *Enhydrobacter* (63) all had a significant negative effect on antibody levels (Table 4). Inclusion of mean log-transformed pre-vaccination antibody levels in the model did not affect these results.

DISCUSSION

Here, we present an overview of nasopharyngeal microbiota profiles of indigenous Venezuelan children and associations with RTIs, GIIs, and post-PCV13 antibody levels.

The most abundant OTU in our cohort of Venezuelan Amerindian children was *Moraxella*, with 40% of children displaying a *Moraxella*-dominated profile. This finding is in line with those from other studies in both Western and tropical populations that included children of comparable age [17, 23–25]. Likewise, the top 5 most common genera in our study population (ie, *Moraxella*, *Corynebacterium propinquum*, *Dolosigranulum*, *Haemophilus*, and *Streptococcus*) are commonly observed in Western populations [2, 3, 17]. However, the top 15 most abundant OTUs in our study contained several bacteria that are not commonly observed in developed regions, including multiple *Acinetobacter* species. Interestingly, other studies performed in populations that reside in humid tropical regions also reported *Acinetobacter* presence in the upper respiratory tract of young children [25, 26], most likely because wet environments, such as moist soil, water plants, and seawater, are known environmental reservoirs for *Acinetobacter* [27]. This suggests a location-specific bacterial colonization pattern, which might have important implications for preventive and therapeutic strategies.

Microbial community composition differed significantly between RTI groups. For example, *Corynebacterium* was overrepresented in children without RTIs, similar to studies from Europe and the United States [17, 28, 29]. Interestingly, several of the bacteria that were associated with the absence of RTIs included environmental bacteria not commonly observed in developed regions such as *Acinetobacter*, *Caulobacteraceae*, and *Pseudomonas*. A recent study in young Fijian children

Table 3. Bacteria Discriminative for Gastrointestinal Infection Groups as Identified by metagenomeSeq and Random Forest

Operational Taxonomic Unit	Log Fold Change	PValue	Adjusted PValue	Method ^a
Associated with disease (GII)				
<i>Klebsiella</i> (7)	−7.20	<.0001	<.0001	Both
<i>Leuconostoc</i> (9)	−4.20	<.0001	<.0001	MGS
<i>Lactobacillus algidus</i> (34)	−3.36	<.0001	<.0001	MGS
<i>Vagococcus</i> (13)	−3.33	<.0001	<.0001	MGS
<i>Staphylococcaceae</i> (101)	−2.81	<.0001	<.0001	MGS
<i>Pedobacter</i> (30)	−2.32	<.0001	.0001	MGS
<i>Thermus thermophilus</i> (26)	−2.00	.0017	.0096	MGS
<i>Anoxybacillus</i> (33)	−1.88	<.0001	<.0001	MGS
<i>Shewanella</i> (69)	−1.58	<.0001	<.0001	MGS
<i>Flavobacterium</i> (14)	−1.23	.0065	.0235	MGS
<i>Vagococcus</i> (20)	−1.21	.0052	.0206	MGS
<i>Myroides</i> (38)	−1.14	.0013	.0080	MGS
Associated with health (absence of GII)				
<i>Acinetobacter</i> (10)	5.06	<.0001	<.0001	MGS
<i>Aerococcus</i> (42)	3.68	<.0001	<.0001	MGS
<i>Acinetobacter</i> (47)	3.14	<.0001	<.0001	MGS
<i>Acinetobacter soli</i> (41)	2.34	<.0001	<.0001	MGS
<i>Wautersiella</i> (61)	2.15	<.0001	<.0001	MGS
<i>Moraxella</i> (1)	1.77	.0120	.0409	MGS
<i>Haemophilus</i> (4)	1.72	.0172	.0513	MGS
<i>Corynebacterium propinquum</i> (2)	1.71	.0165	.0502	MGS
<i>Bergeyella</i> (50)	1.61	.0035	.0164	MGS
<i>Psychrobacter</i> (8)	1.52	.0333	.0858	MGS
<i>Cloacibacterium</i> (94)	1.48	<.0001	.0000	MGS
<i>Helcococcus</i> (22)	1.48	.0234	.0634	MGS
<i>Schlegelella</i> (40)	1.45	.0003	.0023	MGS
BD1 5 (35)	1.39	.0232	.0634	MGS
<i>Actinobacillus porcinus</i> (37)	1.39	.0051	.0206	MGS
<i>Moraxella</i> (59)	1.29	.0059	.0220	MGS
<i>Lysobacter</i> (103)	1.23	.0002	.0015	MGS
<i>Stenotrophomonas maltophilia</i> (58)	1.19	.0307	.0808	MGS
<i>Rothia</i> (32)	1.16	.0053	.0206	MGS
<i>Veillonella</i> (70)	1.05	<.0001	.0003	MGS
<i>Alloprevotella</i> (54)	1.05	.0053	.0206	MGS
<i>Veillonella</i> (62)	1.03	.0014	.0082	MGS
<i>Moraxella</i> (60)	1.00	.0162	.0502	MGS

P values have been adjusted using Benjamini-Hochberg correction for multiple testing.

Abbreviations: GII, gastrointestinal infection; MGS, metagenomeSeq.

^aBoth methods adjusted for age.

residing on a tropical island also reported *Pseudomonas* to be abundantly present in the nasopharynx of healthy children [26]. Like *Acinetobacter*, *Pseudomonas* is present in watery environments, particularly in rivers during the period just before heavy rainfalls that lead to an influx of water into the river [30]. This is in line with the timing of sampling in our study, that is, in the beginning of the rainy season. Although the presence of these bacteria in nasopharyngeal samples could represent environmental contamination, our stringent decontamination procedures and the association of these environmental bacteria with health (absence of infections) suggest that they become part of the resident respiratory microflora in children in tropical watery areas.

Several bacterial taxa were overrepresented in children with RTIs, such as *Klebsiella* and *Actinomyces*. These bacteria are commonly found in immunocompromised patients or populations with chronic lung disease [31–33]. The high prevalence of bronchiectasis caused by recurrent RTIs in indigenous children [34] might have led to alterations in microbiota profiles and facilitation of overgrowth by pathobionts. In addition, poverty, malnutrition, and infectious diseases may have weakened the children's immune systems [35], thereby facilitating colonization by opportunistic bacteria. However, due to the cross-sectional design of our study, it was unknown whether host and environmental characteristics affected bacterial colonization or whether specific bacteria such as *Klebsiella* affected disease susceptibility.

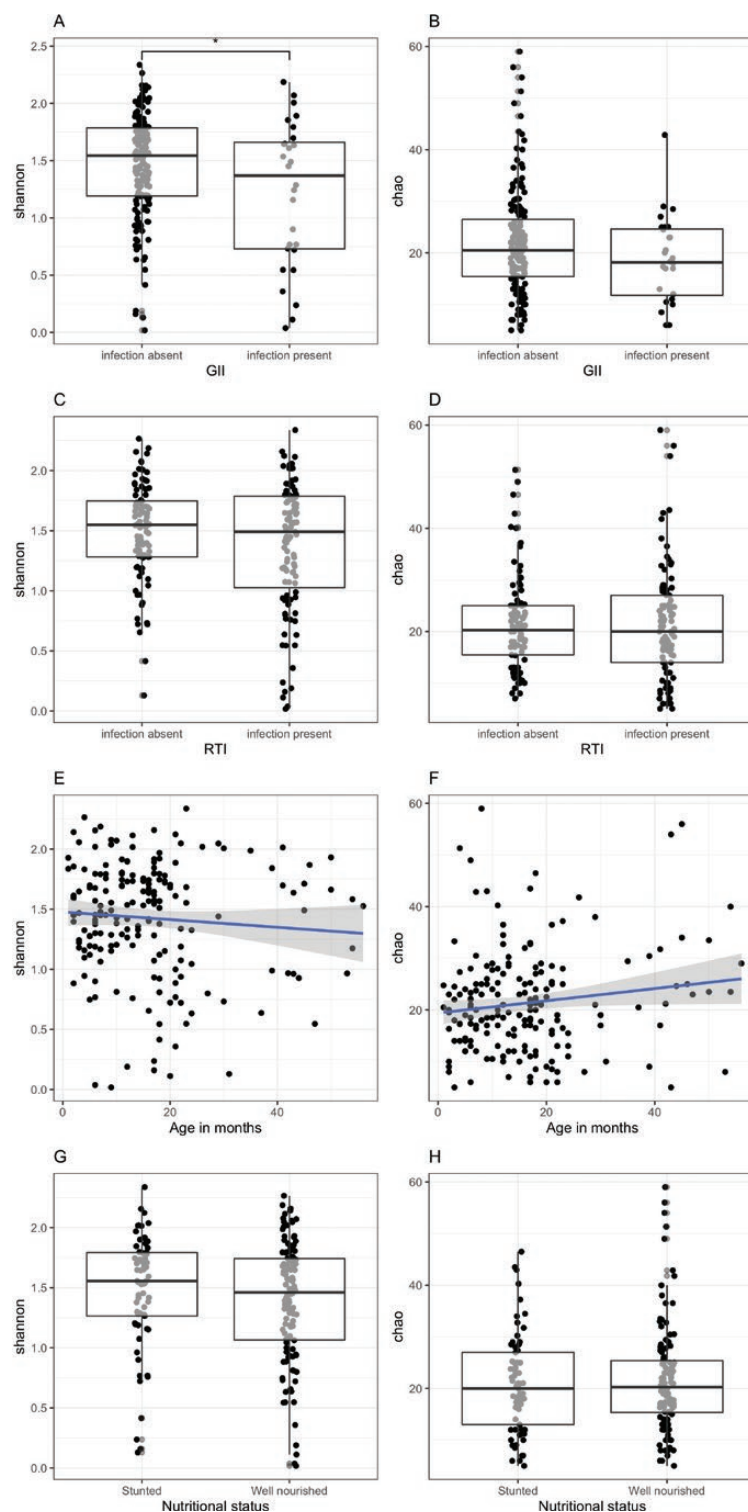


Figure 3. Comparison of Shannon diversity (left panel) and Chao1 index (right panel) split by presence of GII (A and B), presence of RTI (C and D), across age (E and F), and nutritional status (G and H). Abbreviations: GII, gastrointestinal infection; RTI, respiratory tract infection.

Longitudinal studies showed that the combination of environmental triggers and an aberrant nasopharyngeal microbial developmental trajectory may predispose children to RTIs [17, 36].

While previous studies also pointed toward the link between the respiratory tract microbiota and RTIs [17, 26, 36],

disturbances related to GIIs have been much less studied. We observed significant differences in the upper respiratory tract microbiota of children with symptoms of a GII. In fact, the association between bacterial community composition and infection was stronger for GIIs compared with RTIs. While this

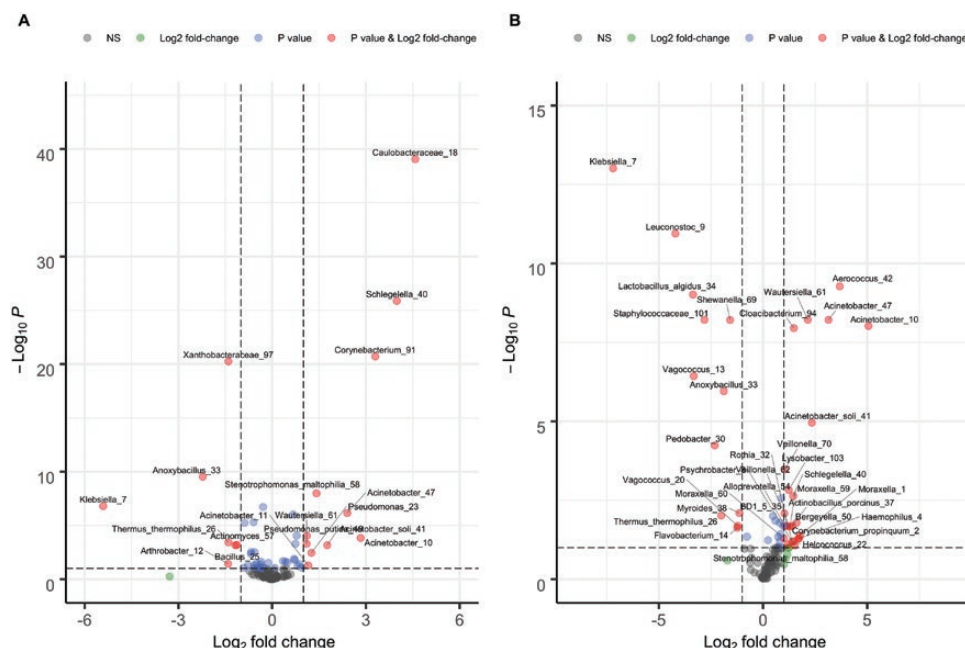


Figure 4. Volcano plot depicting differential abundant operational taxonomic units (OTUs) for (A) respiratory tract infection groups and (B) gastrointestinal infection groups. Bacteria that are overrepresented during disease are depicted on the left (\log_2 fold change [FC] <0). Bacteria that are underrepresented during disease are depicted on the right (\log_2 [FC]>0). Grey dots represent OTUs that were not differentially abundant; blue dots represent OTUs that were differentially abundant but with a \log_2 (FC)<1; green dots represent OTUs that had a \log_2 (FC)>1 but were not significantly differentially abundant; and red dots represent OTUs that were significantly differentially abundant and had a \log_2 (FC)>1. Abbreviation: NS, non-significant.

suggests that respiratory microbiota patterns are related to gastrointestinal symptoms, most research up until this point has focused on the association between intestinal microbiota profiles and respiratory diseases. For example, in studies that

Table 4. Results from Linear Mixed Effect Model on Postvaccination Serum Antibody Levels for Each Covariate

Model Covariate	Estimate (\pm Standard Error)	P Value
Days between vaccination and follow-up	-0.03 (0.01)	<.0001***
Age	-0.03 (0.01)	.0002***
Nutritional status		
Well-nourished vs stunted	-0.27 (0.15)	.08
Breastfeeding		
Breast-fed vs non-breastfed at the moment of sampling	-0.33 (0.21)	.13
<i>Enhydrobacter</i> 63	-0.24 (0.07)	.001**
<i>Arthrobacter</i> 12	-0.19 (0.07)	.01*
<i>Bacillus</i> 25	-0.17 (0.09)	.07
<i>Acinetobacter</i> 11	-0.08 (0.07)	.27
<i>Ornithobacterium</i> 6	-0.08 (0.07)	.30
<i>Moraxella</i> 1	-0.06 (0.08)	.43
<i>Pseudomonas syringae</i> 27	-0.06 (0.09)	.51
<i>Streptococcus</i> 5	-0.04 (0.08)	.65
<i>Sphingobacterium faecium</i> 16	-0.01 (0.08)	.90
<i>Haemophilus</i> 4	-0.004 (0.08)	.95

The estimate reflects the increase or decrease in log mean antibody concentration following 1 unit of change in the predictor variable. Bold values denote statistical significance at the $P < 0.05$ level.

*** $P < .001$, ** $P < .01$, * $P < .05$.

included neonates, gut microbial dysbiosis in early life was associated with asthma at a later age [6, 7]. Our findings point toward a link between the respiratory microbiota and gastrointestinal disease. Gastroenteritis/diarrhea is the most common cause of mortality in our study population who live under precarious sanitary conditions, responsible for almost two-thirds of reported childhood deaths among live births [11]. If GIIs also negatively affect respiratory microbiota diversity in rural populations, this is worrisome since loss of diversity has been associated with respiratory disease severity and the overrepresentation of pathogenic bacteria [17, 37].

Vaccination against respiratory pathogens, including pneumococcal vaccination, has made an enormous contribution to global health [38]. The awareness of the immune-modulating effects of the human microbiota has begun to increase our understanding of its role in vaccine effectiveness [39]. However, again most studies focus on the gastrointestinal microbiota. One of the unique aspects of our cohort is that respiratory microbiota samples were collected before administration of the first PCV13 dose, while serum samples were collected after vaccination. An observed association between prevaccination bacterial taxa and postvaccination antibody response could thus point toward a causative effect of respiratory microbiota on PCV13 antibody response. However, we only observed a significant negative association between 2 bacterial taxa, *Arthrobacter* and *Enhydrobacter*, and antibody response, precluding firm conclusions on the role of the respiratory microbiota in modulating

vaccine response. Interestingly, *Arthrobacter* presence dominated 1 of the 9 community profile clusters, underpinning the fact that this is a bacterial taxon to which rural Warao children are heavily exposed. In line with previous reports, we showed a significant effect of age on antibody response, with older children showing lower antibody levels, and a trend toward higher antibody responses in children compared with well nourished children [15].

In conclusion, this is the first explorative study of the nasopharyngeal microbiota of a large cohort of Venezuelan indigenous children living under extremely remote conditions. In addition to well-known colonizers of the upper respiratory tract, such as *Moraxella* and *Streptococcus* spp., less commonly observed Proteobacteria such as *Klebsiella* and *Acinetobacter* were part of the respiratory microbiota in these Amerindian children, possibly related to their living environment that may favor soil bacteria thriving in this humid area. From a public health perspective, the impact of RTIs and GIs on morbidity and mortality in rural childhood populations is substantial. Our study contributes to a better understanding of the relationship between bacterial carriage and disease susceptibility, which has important implications for prevention and management of infectious diseases in vulnerable childhood populations.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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APPENDIX

STUDY POPULATION

The original study included 504 children aged between 6 weeks and 59 months [16]. The data in the study presented here included samples from a subset of 209 children, mostly (85%)

children aged <2 years. Exclusion criteria were a confirmed or suspected immunosuppressive or immunodeficient condition, including human immunodeficiency virus and major congenital malformations.

Vaccine coverage rates in this population are low; less than one-third of children aged <5 years are fully immunized with the World Health Organization Expanded Program on Immunization vaccines [9]. None of the children had been vaccinated against pneumococcus upon inclusion. Following inclusion and nasopharyngeal sampling, all children were given the primary series of pneumococcal conjugate vaccine vaccination following Centers for Disease Control and Prevention guidelines [40]. This consisted of 3 vaccine doses for children aged 6 weeks–6 months, 2 doses for children aged 7–23 months, and 1 dose for children aged 24–59 months.

DNA EXTRACTION AND SEQUENCING

Bacterial DNA was isolated from 300 µL of sample media in which swabs were stored. Bacterial DNA was extracted using a phenol/bead-beating and a magnetic bead separation method using reagents from LGC Genomics (LGC Limited, England). Only samples that showed a DNA concentration of ≥0.3 pg/µL above negative controls measured using reverse transcription quantitative polymerase chain reaction were sequenced (Supplementary Figure 1). Following DNA extraction, the hypervariable V4 region of the 16S rRNA gene was amplified using bar-coded primer constructs described in [41]. Amplicons were pooled equimolarly following PicoGreen quantification (Thermo Fisher). Library pools were sequenced as 2 × 250PE sequencing runs on the Illumina MiSeq platform (Illumina Inc, San Diego, CA) using the Illumina MiSeq Reagent Kit v2 (500 cycles).

BIOINFORMATIC PROCESSING

Raw reads were processed in our bioinformatic pipeline, which included quality-based trimming of paired-end reads (sickle [42]), assembly (PANDAsq [43]), chimera removal (UCHIME [44]), and clustering into 97% similarity operational taxonomic units (OTUs) and species annotation (SILVA v119 database [45]) as previously described. All but 1 sample (n = 208) fulfilled our quality control standards for reliable analyses. We generated an abundance-filtered dataset by including only those OTUs that were present at or above a confidence level of detection (0.1% relative abundance in at least 2 samples) [46].

OTU CLEANUP PROCEDURES

We included 2 types of negative controls in this dataset, one being skim milk tryptone glucose glycerol (STGG) medium that was taken to the Orinoco Delta but not used for sampling (media controls, n = 4) and the other being nuclease-free water

passed through the laboratory pipeline (technical controls, $n = 12$). We also included bacterial mock communities ($n = 2$) as positive controls (Supplementary Figure 2). Since STGG is a preservative medium optimized for recovery of respiratory pathogens [18], it is widely used in nasopharyngeal carriage studies [47]. It is, however, possible that storage in STGG medium permitted selective overgrowth of certain taxa, despite transportation of samples on ice. To identify selective overgrowth, we followed a 3-step OTU cleanup approach.

First, we identified OTUs with a particularly high read count in the STGG medium samples. We discovered that only 4 of the 140 OTUs in any of the media had a read count of >1000 . Those 4 OTUs were *Pseudomonas lundensis*, *Lactococcus*, *Shewanella*, and *Sphingomonas* (see Supplementary Table 1 for their read counts), which are all known contaminants and appeared to be overrepresented in the controls media. We therefore excluded those OTUs from the data (Supplementary Table 2) [48].

Second, we identified for each remaining OTU in the media controls their highest read count across all media control samples and subtracted this value from the OTU read count in the samples. In doing so, we removed the proportion of reads within a sample that might have originated from the storage medium. In cases where this left a sample with a negative read count for a particular OTU, we replaced it with zero. For example, if the read count for *Staphylococcus epidermidis* was 8 reads in a particular sample, the remaining reads after subtracting the highest read count of *S. epidermidis* in the media (18 reads) would be -10 , in which case the read count for this OTU would be set to 0 in this sample. Following this procedure, we eliminated 3 OTUs that ended up having a total library size of 0 (see Supplementary Table 2).

Third, we identified all OTUs that showed a significant positive correlation between log-transformed raw read counts of an OTU within samples and total log-transformed bacterial density within samples. A significant positive correlation between read count and bacterial density with a slope of > 0.2 indicates that the OTU is highly abundant in high-density samples, which are most likely to be affected by overgrowth. Adopting this strategy, we found 7 potentially overgrowing OTUs that were subsequently removed from the OTU table (Supplementary Table 2).

We further used the Decontam R package to identify possible contaminants using the frequency method [21]. Supplementary Table 2 provides an overview of excluded OTUs at different steps of the cleanup process. To avoid OTUs with identical annotations, we refer to OTUs using their taxonomical annotations combined with a rank number based on the abundance of each given OTU.

Last, all samples with <100 reads after removal of potential contaminating and selective overgrowing OTUs were excluded from the dataset ($n = 11$). In addition, Good's estimator of coverage, that is, the proportion of nonsingleton OTUs in the sample, was calculated to evaluate the sequence coverage. There

were 6 samples with $<99\%$ coverage, which were also excluded, leaving 191 samples available for analysis. Supplementary Figure 3 illustrates the distribution of samples with fewer than 1000 reads across all community profiles, highlighting that there are no cluster profiles that are unique to low-read samples.

STATISTICAL ANALYSES

Alpha Diversity

Alpha diversity was measured using the Shannon diversity index and Chao1 estimate of richness (vegan package in R [49]). The first takes into account both richness and distribution (evenness) of the bacterial species within samples, while the latter takes into account abundance of bacterial species. To assess whether alpha diversity was influenced by clinically relevant variables, we ran linear models with either the Shannon index or the Chao1 estimate as the response variable and with presence of a gastrointestinal infection (GII; factor: infected or uninfected) or presence of an respiratory tract infection (RTI; factor: infected or uninfected) as the determinant. We also accounted for age (continuous) and nutritional status (factor: well-nourished or stunted) in the same models.

Beta Diversity

We grouped together children who displayed similar bacterial community composition using hierarchical clustering based on Bray-Curtis dissimilarity. We used a combination of the Caliński-Harabasz measure and Silhouette index to assess the optimal number of clusters the data should be grouped into. The Fisher exact test was used to find associations between clusters and RTI or GII. We used permutational multivariate analysis of variance (PERMANOVA, vegan, function `adonis2` in R) to assess whether microbiota compositions differed between children who displayed either an RTI or GII. In the model, we also accounted for age, antibiotic use, breastfeeding, and nutritional status with community as a strata. We used nonmetric multidimensional scaling to visualize the difference in microbial community composition between children with or without RTI and with or without GII.

Biomarkers of RTI and GII

In order to identify OTUs that could serve as biomarkers for either RTI or GII, we performed differential abundance analysis using the `fitZig` function (metagenomeSeq R package [22]) accounting for age (continuous). This function fits a zero-inflated Gaussian mixture model to each feature (OTU) and calculates the fold change (on log2-scale) between yes/no RTI or yes/no GII infection groups. *P* values were corrected within the function using Benjamini-Hochberg correction to account for multiple testing. We used an adjusted *P* value of .1 and log2 fold change of 1 as cutoffs for the identification of significant biomarkers. We further validated the results from metagenomeSeq using random forest analysis (VSURF), with 100 trees per forest.

Microbiota Association With Pneumococcal Vaccine Response

To identify host and microbiota factors that influence vaccine response, we first ran a random forest model combining OTUs with host covariates. The host covariates included in the random forest model were recent antibiotic use (factor: used or not used), breastfeeding (factor: breastfed at time of sampling or not), age (continuous), gender (factor: male or female), chronic nutritional status (continuous: height-for-age Z score), population community (factor: Araguabisi, Araguaimuio, Arature, Bonoina, Guayaboroina, Ibaruma, Jobure de Curipao, Merejina, or Winikina), presence of

GII, presence of RTI, and time from primary series completion until blood sampling (continuous: number of days). From this random forest model, we selected the top 15 descriptive variables (OTUs and host variables) to be subsequently used as covariates in a linear mixed-effect model to determine which ones were significantly associated with postvaccination antibody response. Because OTU variables contained a large number of zeros, we performed centering (subtract mean OTU abundance from observations) and scaling (divide observations by OTU standard deviation) to align them with the other model covariates.